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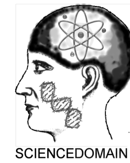

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Comparison of Widal Test with Cultural Methods for Evaluation of *Salmnella typhi/paratyphi* Infection

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Authors' contributions

This work was carried out in collaboration between all authors. Author CIE designed the study, performed the statistical analysis, wrote the protocol and the first drafts of the manuscript and managed literature searches. All the authors CIE, CUI and IPU managed the analysis. All the authors read and approved the final manuscript.

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Original Research Article

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ABSTRACT

Aims: To compare the effectiveness of the Widal screening, stool and blood cultures methods for typhoid/paratyphoid fever diagnosis in exposed subjects.
Study Design: Analysis involved a prospective study.
Place and Duration of Study: The study took place at the Department of Medical Microbiology, University of Nigeria, Enugu Campus, between August 2009 - October 2010.
Methodology: A total of 1000 (500 stool and 500 blood each) samples from 248 males and 252 female aged, < 1 yr-68 yrs. The isolation from samples and identification were carried out with standard methods. Characterization was done using polyvalent sera and confirmed using single factor sera. The rates of recovery of *S. paratyphi* A, B, C and *typhi* D from stool and blood cultures of individuals positive at the various Widal antibody titres were evaluated.
Results: Among the subjects positive for *S. paratyphi* A at 1:160 Widal titre of O and H variants, the isolation rate from stool was 37.5%, while at 1:80 titre 39.1% was isolated from both blood and stool of each subject combined. For those positive for *S. paratyphi B* at 1:160 positive titres, the isolation rates from from stool and blood sample cultures combined was 46.7%. The 1:320 positive

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titre for *paratyphi C* had peak isolation rates (41.7%) from blood samples singly. In the case of *S. typhi D* the peak isolation rate (47.4%) was from blood at titre of 1:320. There was however significant difference (P= .001) among the rates of isolates of *S. typhi* D from blood, stool and stool/blood of subjects with the difference titre. However, there was no correlation (r= .256, .309 and .235), respectively among the data

Conclusion: Blood culture is a major aid to recovery and isolation of the organism in early stages of infection. Stool culture, though not very specific, may also be used as diagnostic method. Widal test, though only a screening method is also sensitive but only results yielding high titre are reliable.

Keywords: Widal test; stool culture, blood culture; Salmonella infection.

1. INTRODUCTION

Salmonella enterica enterica serovar typhi is the causative organism of typhoid fever while *paratyphoid* fever is caused by *Salmonella paratyphi* A, B, or C. These diseases are collectively called enteric fever. Typhoid and paratyphoid fevers, which are diseases associated with poor personal and environmental hygiene, have been controlled in many parts of the world by improved sanitation, but still pose a health threat in some developing nations [1]. Incidences of enteric fever can be used as a measure for the environmental sanitation condition in a region or country. Symptoms of paratyphoid fever are similar to those of typhoid fever but are less severe.

In spite of modern developments, the diagnosis of typhoid fever in many of the developing nations is still being made by the clinicians based on signs and symptoms expressed by the patients. This can result in misdiagnosis, since the signs and symptoms of typhoid/paratyphoid fever may resemble that of other febrile illnesses. However, Widal test is apparently the only typhoid diagnostic technique available in most tropical regions because it is relatively cheap [2]. Notwithstanding the inconsistent laboratory test results obtained in endemic areas, this method is still considered valuable for screening large number of samples; but it requires standardization of reagents and quality assurance [3]. The definitive diagnosis of typhoid fever is by the isolation of *S. typhi* from blood, bone marrow or a specific anatomical lesion [3]. Thus, blood and bone marrow aspirate cultures are the gold standards for the diagnosis of typhoid fever. Although bone marrow cultures are more sensitive, they are difficult to obtain, relatively invasive, and of little use in Public Health settings. Advantageously, the organism can be cultured from stool throughout the duration of disease; though this is not as specific as blood culture. Widal test although robust and simple to perform, is low in both sensitivity and specificity; and reliance on it alone in typhoid endemic areas may lead to misdiagnosis due to either false positive or false negative results [3,4]. However, Widal test is generally accepted as an adjunct to clinical diagnosis and isolation method. For application of proper and adequate measures and public health planning and control it has become necessary to accurately determine the prevalence of the *Salmonella* hence this study is aimed at evaluating and finding out the effectiveness of this commonly used methods.

2. MATERIALS AND METHODS

2.1 Subjects

Samples were obtained from 500 exposed subjects living in the endemic area. Criteria for selection of subjects included the provisional diagnosis of typhoid by the clinician and the symptoms expressed by the subjects. The common symptoms considered in the selection were persistent fever, headache and abdominal disturbances. A total of 500 blood and stool samples each were obtained from randomly selected subjects. The ages of subjects ranged from < 1 year to 68 years. The ethical clearance for the sampling of subjects was obtained from University of Nigeria ethical committee.

2.2 Samples Collection and Processing

Blood were drawn aseptically using 10 ml syringe and needle. About 8 ml of blood were collected from adult and 1-3 ml from each child into vacutainner (plain sterile 15 ml volume stopper glass tubes). The blood was allowed to clot and after centrifugation the serum was separated from the clot. The serum was used for serological screening while the clot was poured into sterile universal containers and minced with scissors. About 2 mls of bile salt broth was added to the clotted blood and incubated. Each tube was

carefully labeled with the patients number already indicated in the laboratory request forms.

For collection of stool samples the subjects were provided with clean wide-mouthed containers with screw covers or sterile containers with wooden spatula. Peanut-sized stool specimens were introduced into these and taken to the laboratory without delay.

2.3 Widal (Qualitative) Slide Agglutination Test Method

Commercially prepared stocks of the Widal agglutination kits were stored at 2-8ºC in the refrigerator. Prior to use, the antigens and the controls were brought out and stood on the bench to attain a steady room temperature. The vials were re-suspended by aspirating dropper several times to obtain a thorough mixing. A drop of serum was placed into each row of circles on paper cards. One drop of each of the negative and positive control sera were dispensed unto two additional circles. One drop of the appropriate antigen suspension was added to each circle next to the sample to be tested. Disposable stirrers or applicator sticks were used to mix and spread over the entire area enclosed by the ring, using separate stick for each mixture. Next, the mixtures were rocked gently on the card for a period of two minutes. The reactions were observed immediately under a suitable light source for any degree of agglutination as positive reaction.

2.4 Quantitative Widal (Tube Agglutination) Test

Six clean plain tubes were placed in racks in two rows and 0.9 ml of normal saline (0.85% sodium chloride) was delivered into the first tube in each row and 0.5 ml into others. Next, 0.1 ml of the test serum was delivered into the first tube and mixed. 0.5 ml volume of the diluted serum from the first tube was delivered into the second tube to obtain two-fold dilution. This procedure was repeated up till the last tube (tube 6) and 0.5 ml of the diluted serum was discarded from tube 6. (At this stage the serial doubling dilution of 1 in 20 to 1 in 640 was obtained). Similar set-up was prepared for the positive and negative control sera then 0.5 of appropriate antigen suspension were added to each. The two racks were shaken carefully to mix the antigens and the sera. Experiments with somatic O antigen were incubated at 50ºC for 4 hours in water bath while those containing the flagella H antigen were incubated for 2 hours in the same temperature. After incubation the result of control tubes were read first by examining the pattern of the sediment and then shaking the tubes gently. Negative reactions showed no visible clumping. Suspension also showed typical swirl when the tubes were flicked. The positive results showed partial or complete agglutinations with variable degree of clearing of the supernatant fluid. The highest dilution of the sera in which agglutinations occurred was taken as titres for the positive reactions. When there was no agglutination the result was recorded as negative or <1:20 titre.

2.5 Bacterial Isolation

The isolation of *Salmonella* organism from blood was made by clot culture technique instead of standard or conventional cultural techniques. The clots were lysed by mincing with sterile scissors to free any organisms trapped in the clot. About 5 ml of the prepared bile salt broth were dropped into each of the containers. These were incubated overnight at 37ºC for 18 to 24 hours. After these subcultures were made from each of the containers unto freshly prepared and dried *Salmonella* Shigella Agar (Biotec) and incubated at 37ºC.

The stool samples were first inoculated into the enrichment medium (Selenite–F broth) and after incubation for 24 hours at 37ºC each was sub-cultured into *Salmonella*-Shigella agar. The SSA plates were incubated overnight at 37ºC and examined for growth.

2.6 Identification of Isolates

Nutrient broth (Lab.M) was prepared. Bijou bottles containing the basal medium and appropriate prepared carbohydrate (mannitol, maltose, dulcitol, sucrose and glucose) were inoculated with drop of the nutrient broth suspension of the test isolate and were loosely capped and incubated at 35ºC overnight. Each was observed for change in colour from amber to red and for gas production (in the medium filled inverted Durham tube).

The test organisms were inoculated heavily on the entire slope surface of the urea agar slants prepared in caped tubes. The tubes were incubated at 37ºC up to 48 hours. Tubes were thereafter examined for change of colour from plain to pink.

Test organisms were inoculated into the triple sugar iron agar (Lab. M) slants contained in test tubes. These were incubated at 35ºC- 37ºC for up to 48 hours. After incubation the TSI agar media were checked for blackening and change in colour from amber to red at the bottom (butt) of the tube.

Suspected colonies were picked and sub-cultured unto moist nutrient agar slopes in MacCartney bottles. These were incubated for minimum of 4 hours.

One to two loopfulls of the agar cultures were mixed with normal saline on clean microscope slides to form a paste. A drop each of the O and H polyvalent sera were added and further mixed with the organisms on the slide. Positive results were indicated by visible agglutination within 30 seconds. Slide tests were repeated for the positive cultures using single factor sera.

2.7 Statistical Analysis

Data were collected, computed and analysed statistically using SPSS, version 15.0. Chi-square formulas were used for two by two contingency tables while the students T-test were used for frequency calculation. Measurement of association between ranks of variables was by correlation test of Kendall rank correlation coefficient. The corelation tests were also carried out to determine the Pearson coefficient (r) value. The values of .05 and .001 properbility were determined at the 95% (5%) and 99.9% (.1%) confidence limits, respectively.

3. RESULTS AND DISCUSSION

The rates of recovery of *S. paratyphi* A from stool and blood cultures of individuals positive at the various Widal antibody titres were evaluated (Table 1). Among the subjects positive for *S. paratyphi* A at 1:80 titre Widal titre of O and H variants, the isolation rate from blood was 31.8%, stool and blood combined was 39.1%. At 1:160 only 8.3% and 20.8% of the subjects were positive for blood and stool cultures singly, respectively and 37.5% of the subjects were positive for the *Salmonella* organism singly at the1 in 160 titre.

For those positive for *S. paratyphi B*, the rates of isolations from blood singly among those positive at various titres were as follows: 1: 20 (0.0%), 1:40 (23.5%), 1:80 (6.7%), 1:160 (6.7%) and 1:320 (50.0%). The isolation rates from by stool and blood sample cultures combined were 46.7% and 50.0% of the 1:80 and 1:160 positive antibody titres, respectively. Singly stool cultures isolates rates (20.0%) were highest from the 1: 160 positive titres (Table 2).

The subjects stool samples singly yielded *Salmonella paratyphi C* isolates with corresponding titres as follows: 1/20 (1.3%), 1/40 (10%), 1/80 (4.3%), 1/160 (27.8%) and 1/320 (16.7%). The 1:320 positive titre for *paratyphi C* had peak isolation rates (41.7%). From blood sample singly and from stool sample singly at 1:160 titre it was 33.3% and 27.8%, respectively. The isolation rate at 1:80 titre was highest (39.1%) from both blood and stool combined (Table 3).

In the case of *S. typhi D* the peak isolation rate (34.6%) was from blood at titre of 1:320. The rate of isolation from blood samples singly at other titres equivalents were as follows: 1: 20 = 7.1%, 1: 40 =10.5%, 1:80 = 7.4% and 1:160 = 17.8%. The highest rate (31.6%) of isolation was by stool singly from subjects with 1 in 320 titre. At 1 in160 titre, the combination of stool and blood

Table 1. Evaluation of subjects for *Salmonella enterica* serotype *Paratyphi* A infection using stool and blood cultures

Antibody titre	NOS.(%) pos at titre (n=500)	NOS.(%) pos by stool culture at titre	NOS.(%) pos by blood culture at titre	NOS.(%) pos by stl/bld culture at titre
1/20	15(3.0)	0(0.0)	0(0.0)	6(2.0
1/40	16(3.2)	1(6.3)	1(6.3)	2(12.5
1/80	22(4.4)	2(9.0)	7(31.8)	13(39.1)
1/160	48(9.6)	18(37.5)	4(8.3)	10(20.8)
1/320	8(1.6)	1(12.5)	0(0.0)	2(25.0)
Total	109	22(20.2)	12(11.0)	33(30.3)
Correlation (R)		0.234	0.040	0.249
P-value		.001	.566	.001

culture of subjects gave an isolation rate of 26.7% (Table 4).

The total rates of *Salmonella* enterica serotypes are shown in Table 5. *Salmonella* Paratyphi A was mostly isolated 67(13.4%). The highest rate (6.6%) was obtained from stool and blood sample combined. However, the highest rate (6.4%) of the *Salmonella typhi* was obtained in the culture of the blood sample. *Salmonella paratyphi* B (2.8%) and paratyphi C (4.8%) were mostly isolated from blood samples singly.

The outcome of Widal reaction for patients with suspected typhoid and malaria depends on the individual immune responses, which becomes stimulated in febrile conditions associated with malaria fever. This memory response could cause positive Widal reactions in previously sensitized patients and accounts for up to 35% of reported false positive Widal test results [5].

Table 2. Evaluation of subjects for *Salmonella enterica* serotype *Paratyphi* B infection using stool and blood cultures

Antibody titre	NOS.(%) pos at titre (n=500)	NOS.(%) pos by stool culture at titre	NOS.(%) pos by blood culture at titre	NOS.(%) pos by stl/bld culture at titre
1/20	8(1.6)	1(0.0)	0(0.0)	0(0.0)
1/40	17(3.4)	1(5.9)	4(23.5)	4(23.5)
1/80	27(27.4)	1(5.0)	5(25.0)	2(10.0)
1/160	15(24.2)	3(20.0)	1(6.7)	7(46.7)
1/320	2(3.2)	0(0.0)	1(50.0)	1(50.0)
Total	62	6(7.5)	11(13.8)	14(17.5)
Correlation		-037	0.110	0.125
P-value		.763	.078	.027

Table 3. Evaluation of subjects for *Salmonella enterica* serotype *Paratyphi C* infection using stool and blood

Antibody titre	NOS.(%) pos at titre (n=500)	NOS.(%) pos by stool culture at titre	NOS.(%) pos by blood culture at titre	NOS.(%) pos by stl/bld culture at titre
1/20	14(2.8)	2(1.3	1(7.1)	4(2.6)
1/40	17(3.4)	0(0.0)	1(5.7)	6(35.5)
1/80	23(4.6)	1(4.3)	2(8.7)	9(39.1)
1/160	18(3.6)	5(27.8	6(33.3)	4(22.2)
1/320	12(2.0)	2(16.7)	5(41.7)	1(8.3)
Total	84	10(11.9)	15(17.9)	24(28.6)
Correlation(R)		0.060	0.116	0.210
P-value		.242	.001	.001

Table 4. Evaluation of subjects for *Salmonella enterica* serotype *Typhi D* infection using stool and blood

Antibody titre	NOS. (%) pos at titre (n=500)	NOS. (%) pos by stool culture at titre	NOS. (%) pos by blood culture at titre	NOS. (%) pos by stl/bld culture at titre
1/20	14(2.8)	0(0.0)	1(7.1)	2(14.3)
1/40	19(3.8)	2(10.5)	2(10.5)	2(10.5)
1/80	34(6.8)	3(8.8)	4(11.8)	6(0.0)
1/160	60(12.0)	9((15.0)	16(26.7)	2(3.3)
1/320	19(3.8)	6(31.6)	9(47.4)	2(10.5)
1/640	2(0.4)	0(0.0)	0(0.0)	0(0.0)
Total	148	20	32	14
Correlation(R)		0.256	0.309	0.235
P-value		.001	.001	.001

A positive Widal test may, therefore, be seen in healthy persons from malaria/typhoid endemic areas as a result of previous sub-clinical infection but in such cases the titre is often low. Widal is not a very accurate method, since patients are often exposed to other bacteria (e.g. *Salmonella enteritidis*, *Salmonella typhimurium*) in this species that induce cross reactivity. Many people have antibodies against these enteric pathogens, which also react with the antigens in the Widal test, causing a false-positive result. This may account for the high rate of positive results with low titre encountered in this research. Therefore, test results need to be interpreted carefully in the light of past history of enteric fever, typhoid vaccination, and general level of antibodies in the populations in endemic areas of the world [6]. The peak isolation rate of *S. Paratyphi A* was 39.1% at 1 in 80 titre. That of *S. paratyphi* B were 50% at 1 in 320 titre. The isolation rate (47.4%) of *S. typhi D* was from blood also peaked at titre of 1:320 (Table 4). These peak isolation rates may be used as rough estimate for the baseline titre for this locality. Baseline value of Widal test result is determined by comparison of efficiency of isolation methods at various antibody titre levels.

The use of blood clot culture, bile salt enrichment broth and highly selective *Salmonella*-Shigella agar to isolate *Salmonella* from blood was highly sensitive and effective. The highly sensitive method of diagnosis employed in this study was a major aid to recovery and isolation of the organism in this early stage of infection even with low Widal titre of 1 in 40 and 1 in 80 (Tables 2 and 4). This goes to confirm that one third of typhoid patients mount no detectable antibody response or no demonstrable rise in titre [7].

There was however significant difference among the rates of isolates of *S. typhi* D from blood, stool and stool/blood of subjects with the difference titre (P= .001). However, there was no correlation among the data (r= 0.256, 0.309 and 0.235, respectively). There was no significant difference in that of isolation of *S. paratyphi A* from blood (P=.556). Blood clot culture is highly sensitive when proper isolation method is used. At Zaria, Nigeria, Florence et al. [8], 22 positives were obtained from Widal test and only 1 isolate from blood culture using thioglycholate broths and MacConkey agar. Similary, at Ebonyi State, Nigeria, using same standard isolation techniques, Nwuzo et al. [9] isolated 2(0.8%) by blood culture against 53(21.2%) obtained by Widal. This inconsistent figures and low numbers of isolates obtained with blood culture may have been as a result of the inappropriate technique used. Blood culture for *Salmonella* is most appropriately carried out using clot culture technique and SSA selective media [10]. Previous antibiotic intake can, however, reduce the chances of isolation of organism from blood culture. This was confirmed by noting that bacteria could be isolated from blood cultures in 73 to 97% of cases before antibiotic use [11]. The organism is obtained from blood during the first week of the illness [3]. The isolation of methods in this study yielded 13.2% *S. typhi*, 13.4% *S. paratyphi* A, 6.2% *S. paratyphi B* and 9.8% *S. paratyphi C* as against that of Ngwu and Agbo [12] in a study carried out around this region in 2003; the distribution was as follows: *S. typhi* 46(34.3%); *S. paratyphi B* 34(25.4%); *S. paratyphi C* 20 (14.9%) and other *salmonella* species 10(25%). This falling incidence shows improvement in sanitation and control measures.

Symptoms of the disease remain positive predictors for choosing which samples to culture in event of scarce resources or large number of samples requiring evaluation. This does not underrate the public health importance value of isolation of the organism from apparently healthy individuals. Most subjects showed no detectable anti-O and/or H antibodies or the antibodies were detected at very low titres for this category of cases that had infections and could only be confirmed by isolation of the *Salmonella* organism from the stool or blood. Colle et al. [13] had reported that about 70% adults formed antibody within the first week of infection. Le Minor [14] further specifically stated that anti-O agglutinins were detected after eight day of the disease while the anti-H agglutinins showed up after 10 days. It is not very clear why individuals harboring the organism could not exhibit detectable antibodies or what factors suppressed expression of antibodies if they were provoked. However, there could be sub-clinical cases at the incubation stage which may later slowly manifest the symptoms with rising antibodies. The significance of these findings lies in the chances of missing these cases when Widal test method is employed exclusively for diagnosis of enteric fevers. Typically, antibodies to O and H antigens start appearing during the end of the first week and peaks at the end of the third week.

Table 5. Total rates of *Salmonella enterica* serotypes from stool and blood samples of subjects

Cultures	*S. paratyphi* A	*S. paratyphi* B	*S. paratyphi* C	*S. typhi* D	Total
Stool	22(4.4)	6(1.2)	10(2.0)	20(4.0)	58
Blood	12(2.4)	11(2.2)	15(3.0)	32(6.4)	70
Stool & Blood	33(6.6)	14(2.8)	24(4.8)	14(2.8)	85
Total	67(13.4)	31(6.2)	49(9.8)	66(13.2)	213

Those positive with stool culture alone is not often reliable. Interpretation of such result clinically may be conflicting. Being that the subjects may be chronic carriers. Most often the carrier's habour the organism in gastrointestinal tract and can be isolated from stool. Such carriers may or may not have had history of typhoid fever. Up to 25% of established chronic carrier cases have no history of clinical enteric fever [7]. Many people become carriers as a result of inadequate medication following diagnosis and self-medication, a practice which is prevalent in Nigeria. However, though stool culture isolations of *Salmonella* are not very specific for clinical cases it may also be used as diagnostic procedure.

4. CONCLUSION

Widal test is an easy and inexpensive test which can be of diagnostic value in situations where blood culture are not obtainable and other automated and sophisticated test are not available as in Nigeria and other African regions. However, the results have to be interpreted with caution considering the base-line titre for each locality also as negative results do not exclude typhoid fever. It is, however, imperative that definitive diagnosis be made by the demonstration of the *Salomonella enterica enterica typhi/paratyphi* in the patients' blood, using appropriate culture techniques.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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